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{Exhibit 47}

Mathews, J.C. and Cormier, M.J., "Rapid Microassay for the Calcium-Dependent Protein Modulator of Cyclic Nucleotide Phosphodiesterase," Methods in Enzymol. Vol LV II, Bioluminescence and Chemiluminescnece, DeLuca, M.A. (Ed.) 107-108 (1978)

Methods in Enzymology

Volume LVII

Bioluminescence and Chemiluminescence

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ACADEMIC PRESS New York San Francisco London 1978
A Subsidiary of Harcourt Brace Jovanovich, Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data
Main entry under title:

Bioluminescence and chemiluminescence.

(Methods in enzymology ; v. 57)
Includes bibliographical references and index.
1. Bioluminescence. 2. Chemiluminescence.
I. DeLuca, Marlene. II. Title.
QP601.M49 vol. 57 [QH641] 574.1'925'08s
[574.1'9125]
ISBN 0-12-181957-4 78-13442

PRINTED IN THE UNITED STATES OF AMERICA

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[11] Rapid Microassay for the Calcium-Dependent Protein Modulator of Cyclic Nucleotide Phosphodiesterase[1]

By JOHN C. MATTHEWS and MILTON J. CORMIER

The activities of certain cyclic nucleotide phosphodiesterases and adenylate cyclases from mammalian heart, brain, and other tissues can be stimulated by an endogenous, low molecular weight, calcium-binding protein when calcium is present.[2-9] The functional species is a Ca^{2+}-modulator protein complex that associates with the phosphodiesterase to increase its biological activity over that of the free enzyme.[4-7] The presence of this protein, referred to here as modulator, has been demonstrated in a variety of mammalian tissues and in extracts of many animal species ranging from coelenterates to mammals.[3-14] It is believed that modulator protein functions in the regulation of cellular cyclic nucleotide levels by providing for rapid modulation of cellular cyclic nucleotide phosphodiesterase activity in response to rapid changes in cellular calcium ion concentration.

This chapter describes a procedure for the rapid, sensitive, and quantitative microassay of modulator protein based on its ability to stimulate

[1] This work was supported in part by National Science Foundation grants (BMS 74-06914 and PCM 76-10573). Contribution No. 365 from the University of Georgia Marine Institute, Sapelo Island, Georgia.

[2] C. O. Brostrom, Y. C. Huang, B. M. Breckenridge, and D. J. Wolff, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 64 (1975).

[3] S. Kakiuchi, R. Yamazaki, and H. Nakajima, *Proc. Jpn. Acad.* **46**, 589 (1970).

[4] Y. M. Lin, Y. P. Liu, and W. Y. Cheung, *J. Biol. Chem.* **249**, 4943 (1974).

[5] J. H. Wang, T. S. Teo, H. C. Ho, and F. C. Stevens, *Adv. Cyclic Nucleotide Res.* **5**, 179 (1974).

[6] Y. Teshima and S. Kakiuchi, *Biochem. Biophys. Res. Commun.* **56**, 489 (1974).

[7] T. S. Teo and T. H. Wang, *J. Biol. Chem.* **248**, 588 (1973).

[8] W. Y. Cheung, *Biochem. Biophys. Res. Commun.* **38**, 533 (1970); W. Y. Cheung, *J. Biol. Chem.* **246**, 2859 (1971).

[9] S. Kakiuchi, R. Yamazaki, Y. Teshima, K. Uenishi, and E. Miyamoto, *Biochem. J.* **146**, 109 (1975).

[10] D. M. Watterson, W. G. Harrelson, Jr., P. M. Keller, F. Sharief, and T. C. Vanaman, *J. Biol. Chem.* **251**, 4501 (1976).

[11] F. C. Stevens, M. Walsh, H. C. Ho, T. S. Teo, and J. H. Wang, *J. Biol. Chem.* **251**, 4495 (1976).

[12] J. R. Dedman, J. D. Potter, and A. R. Means, *J. Biol. Chem.* **252**, 2437 (1977).

[13] J. C. Brooks and F. L. Siegel, *J. Biol. Chem.* **248**, 4189 (1973).

[14] D. Waisman, F. C. Stevens, and J. H. Wang, *Biochem. Biophys. Res. Commun.* **65**, 975 (1975).

ISBN 0-12-181957-4

modulator-deficient phosphodiesterase. The adenosine 5′-monophosphate, produced from adenosine 3′,5′-cyclic monophosphate by the phosphodiesterase reaction, is enzymatically converted to adenosine 5′-triphosphate and assayed with the firefly luminescence reaction, using a modified version of the coupled assay described by Weiss *et al.*[15]

Assay Method

The sequence of reactions involved in the assay of modulator protein is given below.[16]

$$\text{Low-activity PDE} \underset{}{\overset{\text{modulator; } Ca^{2+}}{\rightleftharpoons}} \text{high-activity PDE} \quad (1)$$

$$\text{cAMP} \xrightarrow[Mg^{2+}]{\text{PDE}} \text{AMP} \quad (2)$$

$$\text{AMP} + \text{ATP} \underset{Mg^{2+},\ GTP^{16}}{\overset{\text{myokinase}}{\rightleftharpoons}} 2\ \text{ADP} \quad (3)$$

$$\text{ADP} + \text{PEP} \xrightarrow[Mg^{2+}]{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate} \quad (4)$$

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light} \quad (5)$$

Owing to the requirement for Ca^{2+} in reactions (1) and (2), this portion of the assay must be performed separately since Ca^{2+} inhibits the enzymes in reactions (3)–(5). In addition, since the assay is based on measurement of the initial rate of the luciferase reaction, an incubation period must be allowed for the conversion of AMP to ATP prior to the addition of luciferin and luciferase.

Assay Equipment. A photometer capable of measuring light intensities in the range from 10^7 to 10^{10} photons per second and a strip-chart recorder are required for this assay. Owing to the small volume used in this assay, the photometer must be arranged to collect light from the bottom of small

[15] B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* **45**, 222 (1972).

[16] Abbreviations used are GTP, guanosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; PEP, phosphoenolpyruvate; PP_i, pyrophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; PDE, cyclic nucleotide phosphodiesterase. GTP produces no light with firefly luciferin and luciferase. It is, however, an alternative substrate for myokinase and pyruvate kinase. In this assay, GTP functions as a catalyst in the generation of the initial pool of ATP which is required to perpetuate the cycle.

assay tubes (i.e., 10 × 75 mm).[17] The reader is referred to this volume [41] and [44] for further information concerning suitable photometers and light calibration techniques.

Buffers and Solutions. The following buffers and solutions are required for the assay:

Buffer A: 40 m*M* Tris, 1 m*M* $Mg(Ac)_2$, 1 m*M* cAMP, 0.1 m*M* $CaCl_2$, pH 8.0[18]

Buffer B: 50 m*M* glycylglycine, 15 m*M* DTT, 4 m*M* EGTA, 4 m*M* $Mg(Ac)_2$, 1 m*M* NaN_3, 0.1% w/v BSA, pH 7.8[18]

Solution 1: 10 units of modulator-deficient PDE[19] per milliliter in 40 m*M* Tris, 1 m*M* $Mg(Ac)_2$, pH 8.0

Solution 2: 40 units of myokinase per milliliter, 20 units of pyruvate kinase per milliliter, 10 n*M* GTP, 1 m*M* PEP in buffer B[20]

Solution 3: 0.4 m*M* firefly luciferin plus Sigma firefly lantern extract (8 mg/ml) in buffer B[21]

Preparation of Solution 3. Firefly luciferin is subject to air oxidation so the special precautions outlined here must be taken during its handling to

[17] For unknown reasons this assay is very sensitive to inhibition by contaminants in glassware. The authors recommend that all glassware, especially the assay tubes, be washed with 6 *N* HCl followed by deionized water as the last step.

[18] The characteristics of the assay change markedly as buffer A ages. Therefore, to ensure reproducibility, this buffer should be prepared fresh daily. Buffer B should be prepared fresh each week.

[19] The authors are indebted to Dr. Frank Siegel for kindly providing the purified and lyophilized porcine brain modulator protein and modulator-deficient PDE that were used to develop this assay procedure. Satisfactory procedures for the preparation of modulator-deficient PDE may be found in references cited in footnotes 6–8. Aliquots (125 μl) of solution 1 may be prepared in advance and stored at −80° without significant loss of activity. One unit of PDE is defined as that amount of PDE which is sufficient to produce 1 nmol of AMP per minute when saturated with modulator and Ca^{2+} at pH 8.0 at 25°. One unit of modulator is defined as that amount of modulator required to produce 50% maximal activation of 1 unit of PDE.

[20] Solution 2 must be prepared fresh daily in the amount required, because myokinase and pyruvate kinase are unstable. These enzymes may be purchased as stable suspensions in ammonium sulfate and added to solution 2 directly. One unit of myokinase is defined as that amount of enzyme which is sufficient to produce 1 μmol of ATP per minute at pH 7.5 at 37°. One unit of pyruvate kinase is that amount of enzyme required to produce 1 μmol of pyruvate per minute at pH 7.5 at 37°. A 100 × stock solution of PEP and GTP in buffer B may be prepared weekly and stored at 4°.

[21] Firefly luciferase may be crude or purified without affecting the characteristics of this assay. The pure enzyme was a generous gift from Dr. M. DeLuca. Sigma lyophilized lantern extract is the recommended source of this enzyme owing to its lower cost, more ready availability, and stability in the dry state. The amount of firefly extract employed in solution 3 corresponds roughly to 5×10^{-3} units/ml. One unit of FF luciferase is defined as that amount required to produce 10^{13} protons sec^{-1} at pH 7.8 at 25° in a system containing 0.5 m*M* ATP and 20 μ*M* luciferin.

ensure accuracy. Deoxygenate 5 ml of methanol by bubbling argon through it for at least 5 min. Dissolve approximately 200 μg of D-luciferin in 1 ml of deoxygenated methanol, and maintain this solution under an argon atmosphere. Prepare 1 ml of a 1:10 dilution of the luciferin solution with deoxygenated methanol and place it in a stoppered 1-ml capacity quartz cuvette under argon. Determine the absorption of this solution at 268 and 327 nm. The molar extinction coefficient for luciferin at 327 nm is 18,800, and for luciferin free of oxidation products, the 327 nm/268 nm absorption ratio should be 2.5.[22] However, if this ratio is greater than 1.5 the luciferin is sufficiently pure and the absorption value at 327 nm can be taken as due solely to luciferin. Once the luciferin concentration is accurately known, divide the stock solution into vials so that each vial contains 50 nmol of luciferin. Evaporate the methanol by blowing argon vigorously over the surface of the solution. Store the dried luciferin vials desiccated, in the dark, below 0°. This procedure is sufficient for preparing approximately 14 vials of luciferin. Immediately prior to use, add 1 mg of lyophilized lantern extract and 125 μl of buffer B at 4° to a luciferin vial. This is sufficient luciferin and luciferase for 25 assays. It should be kept at 0°–4° and used within 2 hr of preparation.

Procedure. Step 1: Add 1–5 μl of modulator protein solution (with a chelator concentration < 0.1 m*M*) to 50 μl of buffer A. Initiate the reaction by adding 5 μl of modulator-deficient PDE (solution 1). Incubate for 20 min at room temperature.

Step 2: Pipette 10 μl of the reaction mixture from step 1 into 100 μl of solution 2 in a 10 × 75 mm test tube. Incubate for 5 min at room temperature.

Step 3: Pipette 5 μl of firefly luciferin–luciferase (solution 3) into the reaction mixture from step 2. Mix by gently vortexing, place the tube in the photometer sample compartment and record the initial light intensity. This step should be accomplished within 10 sec of the addition of solution 3.

To ensure that modulator is the factor being assayed, control experiments should be performed with no added PDE. In addition, 1 m*M* EGTA (final concentration) may be added to the assay at step 1, which will prevent the activation of PDE from occurring by removing free Ca^{2+} from the reaction mixture. Interfering amounts of endogenous PDE activity in a modulator sample may be destroyed by heating the samples to 95° for 5 min.

The relationships shown in Figs. 1–3 were determined using the assay method described here. With PDE left out of the complete assay mixture, accurate measurements were obtained for ATP ranging in concentration

[22] E. H. White, F. McCapra, G. F. Field, and W. P. McElroy, *J. Am. Chem. Soc.* **83**, 2402 (1961).

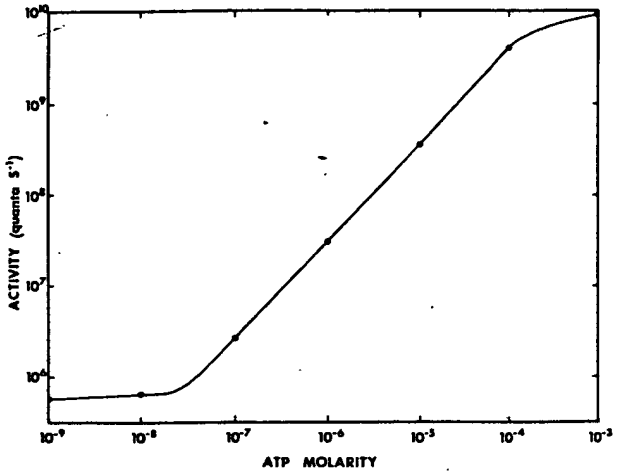


FIG. 1. Dependence of light intensity on ATP concentration in the coupled assay. The data were obtained by adding 10-μl aliquots of known concentrations of ATP in buffer A to step 2 of the assay procedure.

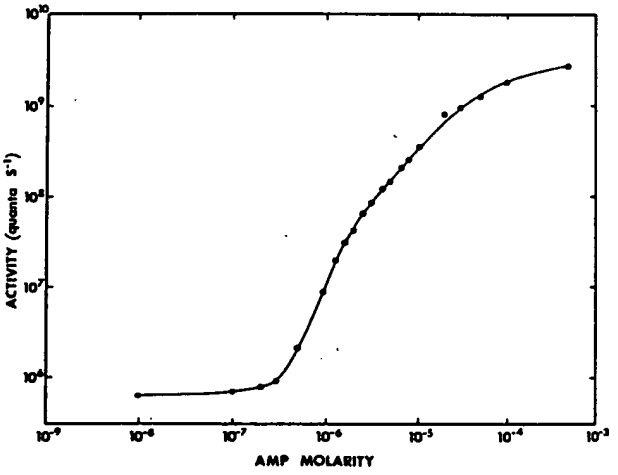


FIG. 2. Dependence of light intensity on AMP concentration in the coupled assay. Data were obtained by adding 10-μl aliquots of known concentrations of AMP in buffer A to step 2 of the assay procedure.

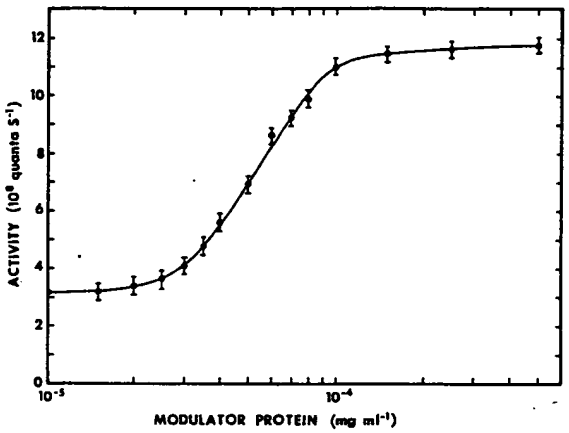


FIG. 3. Dependence of light intensity on modulator protein. Aliquots (5 μl) of known concentrations of porcine brain modulator protein [J. C. Brooks and F. L. Siegel, *J. Biol. Chem.* **248**, 4189 (1973); see also text footnote 19] in buffer A were assayed as described.

from 50 n*M* to 0.5 m*M* (Fig. 1) and for AMP concentrations ranging from 0.5 μ*M* to 0.5 m*M*. (Fig. 2). Modulator concentrations, standardized against porcine brain modulator,[19] were determined over approximately a 10-fold concentration range (Fig. 3). At saturating levels of modulator, a 3- to 4-fold stimulation of the PDE activity was observed.

Advantages of the Luminescence assay. This assay is relatively rapid and, by initiation of steps 1–3 at appropriate time intervals, up to 20 assays can be performed within a 1-hr period. The assay is also sensitive, thus allowing one to conserve material. For example, the amount of PDE used in this assay procedure is approximately 2% of the amount required in more conventional colorimetric assays for PDE.[23] With the exception of modulator-deficient PDE, all reagents used are commercially available.

[23] R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.* **237**, 1244 (1962).

To test lipase or phospholipase activity, the enzymes are incubated with the proper substrate under appropriate conditions; aliquots are taken with time and boiled to inactivate the enzyme, and the liberated myristic acid is assayed. Alternatively, the substrate and the lipase or the phospholipase can be incubated together with the M17 cells. Since as little as 10 pmol of myristic acid can be detected, low enzymic activity can be readily followed within short time periods. Moreover, it is possible to use substrates where myristic acid is linked only to one of the glycerol positions (e.g., the β position), while nonactive fatty acids, such as stearic acid, are linked to the α and α' positions. Using such substrates, one can determine the specificity of the lipase for the α' or β' positions.

When testing lipase or phospholipase activity, one must be alert to the possibility that there may be inhibitory unsaturated fatty acids coming from contaminating triglycerides. One way to control for this is to incubate the lipase or phospholipase without the addition of the myristic acid-containing substrate. Samples are then taken with time from the assay mixture and tested for their effect on the response of M17 cells to known concentrations of myristic acid. When no unsaturated fatty acids (or other inhibitors) are present, the response of the M17 cells will be the same in the presence and in the absence of the tested sample.

A bioassay for antilipogenic compounds can also be carried out with this system. Antilipogenic compounds, such as cerulenin[6] and C-55,[7] inhibit bioluminescence,[8] providing the basis for the assay.

[6] A. Omura, *Bacteriol. Rev.* **40**, 681 (1976).
[7] T. Ohno, J. Awaya, T. Kesado, S. Nomura, and S. Omura. *Antimicrob. Agents Chemother.* **6**, 387 (1974).
[8] S. Ulitzur and I. Goldberg. *Antimicrob. Agents Chemother.* **12**, 308 (1977).

[21] The Luciferase Reduced Flavin Hydroperoxide Intermediate

By J. W. HASTINGS and JAMES E. BECVAR

$$FMNH_2 + E + O_2 \longrightarrow E\text{-}FMNH\text{-}OOH$$

$$E\text{-}FMNH\text{-}OOH + RCHO \longrightarrow cq\ (h\nu) + E + FMN + RCOOH + H_2O$$

$$E\text{-}FMNH\text{-}OOH \xrightarrow{\text{spontaneous}} E + FMN + H_2O_2$$

where E = bacterial luciferase; $FMNH_2$ = reduced riboflavin 5′-phosphate; E-FMNH-OOH = the luciferase-bound 4a-peroxy-reduced flavin; cq = bioluminescent quantum yield.

ISBN 0-12-181957-4

Principles

First, the turnover number for luciferase in the presence of saturating amounts of all substances is very low; i.e., the time required for a single catalytic cycle is long,[1,2] namely, 2–20 sec at 20° depending on the luciferase and the chain length of aldehyde used.[3,4] Because of a sufficiently positive enthalpy of activation for the rate-limiting step preceding light emission, turnover (emission) can be effectively prevented by lowering the temperature.[5]

Second, the reaction of oxygen with luciferase-bound reduced flavin occurs in the absence of aldehyde to produce the same intermediate as is involved in the light reaction.[1,6] This bound peroxy reduced flavin intermediate decomposes spontaneously at a relatively slow rate, having a half-life (*Beneckea harveyi*) of about 10 sec at 20°. The high enthalpy of activation for the spontaneous decomposition reaction has the import that, by lowering the temperature (if only to 0°), the intermediate is sufficiently stabilized to allow its chromatographic isolation. In this way, one can obtain an essentially homogeneous solution of enzyme molecules, each of which is poised at the same state in catalysis, a true chemical intermediate in the overall reaction. During the slow spontaneous decomposition, the amount of intermediate remaining at any time can be assayed by mixing an aliquot of the sample with aldehyde and simultaneously increasing temperature.[2]

Materials

Luciferase purified from *Beneckea harveyi*, *Photobacterium fischeri*, or *P. phosphoreum*[7,8]
Sephadex G-25 column (1 × 18 cm)
NaCl, 0.5 *M*, in 0.3 *M* phosphate buffer, pH 7.0 (elution buffer)
Phosphate buffer, 0.1 *M*, pH 7.0 (assay buffer)
Long-chain aldehyde; decanal, dodecanal, or tetradecanal are satis-

[1] J. W. Hastings and Q. H. Gibson, *J. Biol. Chem.* **238**, 2537 (1963).
[2] J. W. Hastings, C. Balny, C. LePeuch, and P. Douzou, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3468 (1973).
[3] J. W. Hastings, J. A. Spudich, and G. Malnic, *J. Biol. Chem.* **238**, 3100 (1963).
[4] J. W. Hastings, K. Weber, J. Friedland, A. Eberhard, G. W. Mitchell, and A. Gunsalus, *Biochemistry* **8**, 4681 (1969).
[5] J. W. Hastings, Q. H. Gibson, and C. Greenwood, *Proc. Natl. Acad. Sci. U.S.A.* **52**, 1529 (1964).
[6] J. W. Hastings, *Annu. Rev. Biochem.* **37**, 597 (1968).
[7] T. O. Baldwin, M. Z. Nicoli, J. E. Becvar, and J. W. Hastings, *J. Biol. Chem.* **250**, 2763 (1975).
[8] J. W. Hastings, T. O. Baldwin, and M. Z. Nicoli, this volume [14].

factory (10 ml of aldehyde in 10 ml of water dispersed by sonication)
Sodium hydrosulfite (solid)
Flavin mononucleotide, 5 m*M*

Procedure[9]

Isolation procedures can be carried out in a walk-in cold room with cold baths and jackets to maintain temperatures at 0°. A small (1 × 18 cm) Sephadex G-25 column is preequilibrated at 0° with buffer containing 0.5 *M* NaCl in 0.3 *M* phosphate buffer, pH 7.0. A flow rate of between 1 and 2 ml/min is used. To 1 ml of buffer containing about 1 mg of luciferase, an excess (about 2-fold) of FMN is added and then just enough solid sodium hydrosulfite to bleach the solution. This anaerobic mixture is promptly applied to the column and chromatographed, whereupon the enzyme-containing fraction migrates ahead of the hydrosulfite and reaction with oxygen occurs. The intermediate elutes with the void volume, well separated from free low-molecular-weight species such as free oxidized flavin, H_2O_2, and dithionite oxidation products. Larger quantities of enzyme (e.g., 10 mg) can be used.

Chromatography may also be carried out at lower temperatures, down to about −30°, by using a buffer composed of 50:50 ethylene glycol–phosphate buffer.[2]

Assay

The activity is measured by rapidly mixing small aliquots of the intermediate with long-chain aldehyde in buffer at 25°. A sample (0.1 ml) is taken up in a cooled syringe and injected into buffer (1.8 ml, 0.1 *M* phosphate, pH 7) containing aldehyde appropriate in amount and chain length for the luciferase species used (e.g., for *P. fischeri* intermediate, 0.1 ml of 0.1% sonicate of decanal, for final assay concentration of 0.25 m*M*).

Properties

Composition, Stability, and Reactivity. The intermediate as isolated contains about 1 mol of bound flavin chromophore per mole of enzyme.[2] In the absence of aldehyde, the intermediate decomposes to form equimolar amounts of FMN, H_2O_2, and free luciferase at a rate that depends not only on the temperature, but also on the buffer, ionic strength, and type of

[9] J. E. Becvar, S.-C. Tu, and J. W. Hastings, *Biochemistry* 17, 1807 (1978).

luciferase (species of bacteria).[9,10] At 0°, the half-life at high ionic strength can be longer than 30–40 min for the intermediate prepared from *B. harveyi* luciferase; for the *P. fischeri* intermediate, the half-life can be more than an hour. The half-life is shorter at lower ionic strength. For later use, samples may be stored at lower temperatures, either by freezing, or in the liquid state by mixing with ethylene glycol.

In the presence of aldehyde, the intermediate is less stable at lower temperatures and will emit light even below 0°. The products of the reaction in the presence of aldehyde are FMN, RCOOH, H_2O, and light. An important point is that dissolved oxygen is not required for, nor does it affect the rate of, reaction of the peroxy intermediate either with or without aldehyde.[9]

Absorption Spectra. With all luciferases, the spectrum for the isolated intermediate at wavelengths longer than 300 nm exhibits a single absorbance peak in the 370 nm region ($\epsilon \sim 1 \times 10^4 M^{-1}\,cm^{-1}$). Incubation in the absence of aldehyde results in the slow loss of potential for luminescence and at the same time a dramatic absorbance increase in the 450 nm region, producing a spectrum resembling that of oxidized flavin. Three clear isosbestic points occur during this reaction (approximately 329, 368, and 401 nm for the *B. harveyi* intermediate and 333, 382, and 404 nm for *P. fischeri*).[11] The product has been identified as FMN.[13]

Decomposition Kinetics and Light Yield. The loss in luminescence potential and increase in 450 nm absorbance are processes obeying first-order kinetics. Although the rate constant describing both processes is dependent on the temperature, buffer, ionic strength, and type of luciferase used, both kinetic processes occur in parallel at the same rate under all conditions so far examined.

By comparing the bioluminescence capacity remaining in the sample with the amount of flavin chromophore still remaining in the intermediate form, quantum yields of 0.19 and 0.27 per flavin have been reported for *B. harveyi* and *P. fischeri* intermediates, respectively.[9]

Fluorescence Spectra. The intermediate of *B. harveyi* exhibits fluorescence emission in the 500 nm region which increases dramatically (about 5-fold) upon irradiation, with an ultimate fluorescence quantum yield equal at least to that of free FMN.[13] Similar fluorescence properties were not evident with the intermediate of *P. phosphoreum.*[14]

[10] J. W. Hastings and C. Balny, *J. Biol. Chem.* **250**, 7288 (1975).
[11] The exact values for the isosbestic points with *B. harveyi* reported in this publication[9] are not in agreement with other observations.[2,12] The reason for this discrepancy is not clear.
[12] J. W. Hastings, *Energy Transform. Biol. Systems, Ciba Found. Symp. 1975.*
[13] C. Balny and J. W. Hastings, *Biochemistry* **14**, 4719 (1975).
[14] N. Ashizawa, T. Nakamura, and T. Watanabe, *J. Biochem.* **81**, 1057 (1977).

[23] Immobilization of Bacterial Luciferase and Oxidoreductase and Assays Using Immobilized Enzymes

By EDWARD JABLONSKI and MARLENE DELUCA

The immobilization of an enzyme generally results in increased stability and the additional property of reusability, while retaining specificity for substrates and effectors. The immobilized enzyme can be as useful an analytical tool as the soluble counterpart, with the added advantages of easier handling and decreased cost. The extreme sensitivity and specificity of the luminescent enzymes from firefly and marine bacteria for the determination of ATP, NADH, and NADPH and for monitoring reactions producing these compounds has been shown in preceding chapters of this volume.

This article describes the immobilization of the NADH and NADPH:FMN oxidoreductases and luciferase from *Beneckea harveyi* onto arylamine glass beads. These immobilized enzymes are individually active and also function to produce light via a coupled reaction utilizing NADH or NADPH.[1]

$$NAD(P)H + H^+ + FMN \rightarrow NAD(P)^+ + FMNH_2 \quad (1)$$

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + h\nu \quad (2)$$

The availability of highly purified oxidoreductases specific for NADH or NADPH has led to the development of an immobilized enzyme system capable of continuous monitoring of reactions producing NADH or NADPH.[2]

Preparation and Immobilization of Enzymes

Bacteria

Beneckea harveyi strain No. 392 (formerly MAV) was obtained from Dr. K. H. Nealson of the Scripps Institute of Oceanography. The cells are grown at 25° in a medium containing, per liter, the following components: 3 ml of glycerol, 30 g of NaCl, 0.1 g of $MgSO_4$, 3.7 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $(NH_4)_2HPO_4$, 5 g of bacto-peptone (Difco), and 3 g of

[1] E. Jablonski and M. DeLuca, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3848 (1976).
[2] E. Jablonski and M. DeLuca, *Anal. Biochem.*, in press.

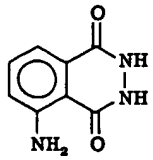
ISBN 0-12-181957-4

[36] The Chemiluminescence of Luminol and Related Hydrazides

By DAVID F. ROSWELL and EMIL H. WHITE

Chemiluminescence is the production of light by chemical reactions. Electronically excited states are formed in the chemical reaction, and light emission from these completes the process. The relationship between chemiluminescence and absorption/emission phenomena, such as fluorescence, are shown in Fig. 1.

A surprisingly large number of chemical reactions are chemiluminescent.[1-4] This paper, however, will deal only with a rather famous example, the chemiluminescence of luminol [5-amino-2,3-dihydrophthalazine-1,4-dione (compound I)]; some derivatives and analogs will also be discussed.



(I)

The first report of chemiluminescence from luminol was made by Albrecht[5] in 1928. Since that time the reaction of luminol and other derivatives of the general hydrazide structure[6] have been studied extensively. A number of reviews on this subject have appeared over the last 15 years,[7-10] and hence the historical aspects of the problem will be treated only briefly.

[1] K.-D. Gundermann, *Top. Curr. Chem.* **46**, 61 (1974).
[2] E. H. White and D. F. Roswell, *Acc. Chem. Res.* **3**, 54 (1970).
[3] M. J. Cormier, D. M. Hercules, and J. Lee, eds., "Chemiluminescence and Bioluminescence." Plenum, New York, 1973.
[4] K.-D. Gundermann, "Chemilumineszenz Organischer Verbindungen." Springer-Verlag, Berlin and New York, 1968.
[5] H. O. Albrecht, *Z. Phys. Chem.* **136**, 321 (1928).
[6] Luminol as well as being named as a phthalazinedione is also referred to as 3-aminophthalic hydrazide.
[7] E. H. White, *in* "Light and Life" (W. D. McElroy and B. Glass, eds.), 1st ed., p. 183. Johns Hopkins Press, Baltimore, Maryland, 1961.
[8] K.-D. Gundermann, *Angew. Chem., Int. Ed.* **4**, 566 (1965).
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ISBN 0-12-181957-4

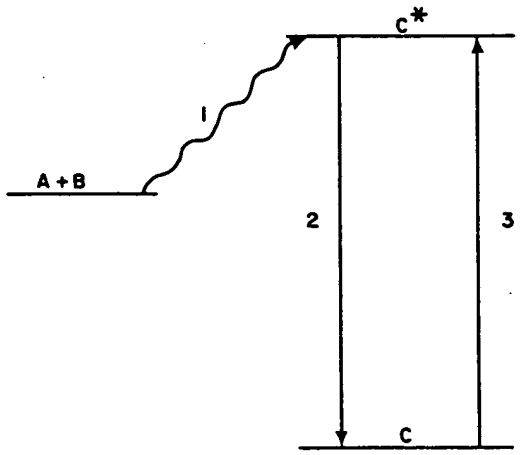
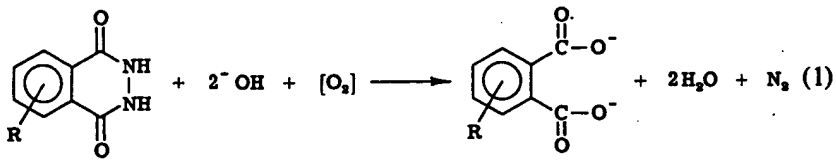


FIG. 1. Chemiluminescence, path 1 + 2; fluorescence, path 2; absorption, path 3.

Reaction Conditions

The chemiluminescent reactions of luminol are all oxidations [Eq. (1)]. A wide variety of reaction conditions have been employed; the reaction can be carried out either in protic media or in aprotic solvents such as dimethyl sulfoxide (DMSO), hexamethylphosphoric acid triamide (HMPT), or dimethylformamide (DMF). In aprotic media, only oxygen and a strong base are required for chemiluminescence.[7]



Water is the most common protic solvent, although the reaction has been studied in the lower alcohols as well. The reaction in these solvents usually requires base, an oxidizing agent, and either oxygen or a peroxide, depending on the system. Hypochlorite, ferricyanide, and persulfate are commonly used oxidizing agents. In place of the oxidizing agents, chelated transition metal complexes, pulse radiolysis,[11] or sonic waves[12] have been used in the presence of either oxygen or peroxides.

[11] J. H. Baxendale, *Chem. Commun.* **1971**, 1490 (1971).
[12] E. N. Harvey, *J. Am. Chem. Soc.* **61**, 2392 (1939).